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Photoaccumulation of two ascorbyl free radicals per photosystem I at 200 K

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Abstract

Illumination of photosystem I (PSI) from the cyanobacterium *Synechocystis* sp. PCC 6803 at 200 K in the presence of ascorbate leads to the formation of two ascorbyl radicals per PSI, which are formed by $P700^+$ reduction by ascorbate. During photoaccumulation, one half of the ascorbyl radicals is formed with a halftime of 1 min and the other half with a halftime of 7 min. Pulsed electron paramagnetic resonance (EPR) experiments with protonated/deuterated PSI show that a PSI proton/deuteron is strongly coupled to the ascorbyl radical. Our data indicate that reactive ascorbate molecules bind to PSI at two specific locations, which might be symmetrically located with respect to the pseudo-C_2 axis of symmetry of the heterodimeric core of PSI. Reduction of $P700^+$ by ascorbate leads to multiple turnover of PSI photochemistry, resulting in partial photoaccumulation of the doubly reduced species (F_A^-, F_B^-). A modified form of F_B^-—in accordance with Chamorovsky and Cammack [Biochim. Biophys. Acta 679 (1982) 146–155], but not of F_A^-, is observed by EPR after illumination at 200 K, which indicates that reduction of F_B at 200 K is followed by some relaxation process, in line with this cluster being the most exposed to the solvent.

Keywords: Photosynthesis; Ascorbate; EPR; HYSCORE; Protein relaxation; Iron–sulfur cluster; Hydrogen bond

1. Introduction

Photosystem I (PSI) is a light-driven transmembrane oxidoreductase located in the thylakoid membranes of higher plants, algae and cyanobacteria. It is a multiprotein complex [1] containing many cofactors involved in light collection and charge separation [2,3]. The three-dimensional structure of PSI from the cyanobacterium *Thermosynechococcus elongatus* has been solved recently at a 2.5-Å resolution [4]. This structure revealed an approximately twofold symmetry involving particularly the two large and similar membrane subunits PsaA and PsaB as well as the cofactors which are bound to these two subunits. This symmetry allows to define two branches of electron transfer sharing the primary electron donor P700, a dimer of chlorophyll *a*, and the secondary acceptor F_X (a 4Fe–4S cluster) which are both located on the pseudo-C_2 axis of symmetry. Between P700 and F_X, each branch contains a primary acceptor A_0 (a chlorophyll *a* monomer), a secondary acceptor A_1 (a phylloquinone) and an accessory chlorophyll *a* located between P700 and A_0. Experimental evidence that both branches are active has been recently provided [5] though this matter is still somewhat controversial [6]. The terminal electron acceptors of PSI are two 4Fe–4S clusters named F_A and F_B, which are both bound to the small peripheral subunit PsaC. F_A lies closer to F_X than F_B whereas F_B is the PSI cluster which is the most exposed to the solvent and is the direct partner of soluble ferredoxin [7].

PSI can undergo charge separation at temperatures down to 4 K as is the case for all kinds of photosynthetic reaction centers. The temperature dependence of charge separation and stabilization follows a complex pattern which has been studied in several laboratories (reviewed in Ref. [8]). Below 77 K, charge separation between $P700^+$ and F_A^- or F_B^- is irreversible, with the relative proportions of photoreduced F_A and F_B varying considerably with samples and experimental conditions. However, this charge separation is in-

Abbreviations: PSI, photosystem I; EPR, electron paramagnetic resonance; DCPIP, (2,6-dichlorophenol)indophenol; P700, the primary electron donor of photosystem I; F_A, F_B, F_X, the [4Fe–4S] clusters of photosystem I

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complete as, in a fraction of the reaction centers, only the reversible photoreduction of the phylloquinone(s) A_1 is observed. This is followed by charge recombination whereas forward electron transfer from A_1 to the first 4Fe–4S cluster F_X is inhibited [9]. Illumination of PSI at 200–220 K has been extensively used as a way of accumulating reduced acceptors under highly reducing conditions, i.e. in the presence of dithionite at pH ≥ 8 (see, e.g. Ref. [10]) This method relies on the fact that dithionite can reduce $P700^+$, at a rate large enough that it can compete with the recombination reactions between $P700^+$ and reduced acceptors. It takes also advantage of the fact that, in these anaerobic conditions, the reduced acceptors cannot be reoxidized by oxygen during the light treatment. For studying PSI photochemistry at low temperature under moderate conditions, ascorbate is generally used to reduce $P700^+$ at room temperature before freezing the sample in darkness, whereas the acceptors are in the oxidized state. When studying such samples, it is generally assumed that no reduction of $P700^+$ by ascorbate is possible at temperatures below 220 K. In the present study, we will show that this assumption is incorrect and that ascorbate can reduce $P700^+$ at 200 K, albeit very slowly, leading to the photoaccumulation of stoichiometric amounts of ascorbyl radicals. Some properties of the ascorbyl radicals will be reported together with some characteristics of PSI associated with the radical formation.

Ascorbate plays an important role in biological systems as a redox component and is considered to be an ubiquitous antioxidant, present, e.g. in human plasma at concentrations of 50–150 μM. It has been found in various cells and subcellular compartments, e.g. in human neutrophils [11], in the cytosol and chloroplast of photosynthetic eucaryotic cells [12] and in mitochondria [13]. It acts as a substrate for many different enzymes such as myrosinase [14], myeloperoxidase [15], ascorbate oxidase [16] and ascorbate peroxidase [17]. As an example to indicating its essential and versatile role, it has been recently proposed to be responsible for the generation of $NO^{\cdot}$ from peroxynitrite in mitochondria [13]. Ascorbate may act as a one- or two-electron redox component. Its redox potentials at pH 8.0 are approximately 230 and −140 mV versus SHE for the $A^{\cdot -}/HA^-$ and $A/A^{\cdot -}$ couples, respectively [18,19]. The ascorbyl free radical is formed during the catalytic turnover of, e.g. ascorbate oxidase and ascorbate peroxidase. Despite the importance of this radical, this report constitutes, to our knowledge, the first example of an ascorbyl radical which could be trapped for low temperature studies in a homogeneous biological environment.

2. Materials and methods

2.1. Biological samples

PSI from the cyanobacterium *Synechocystis* sp. PCC 6803 was prepared as in Ref. [20] following Ref. [21]. Digitonin-solubilized PSI particles from spinach were prepared according to Ref. [22]. Deuterated PSI was isolated from *Synechococcus* sp. PCC 7002 cultivated in A+ medium containing 95% D_2O as described previously [23].

2.2. Electron paramagnetic resonance (EPR) experiments

Continuous wave (CW)-EPR spectra were recorded with a Bruker ER 200D X-band spectrometer equipped with an Oxford Instruments cryostat. The microwave frequency was measured with a microwave frequency counter HP 5350B. Samples were prepared in calibrated tubes of 3-mm internal diameter. Most samples were prepared in Tricine 20 mM pH 8. When present, ascorbate was added at a final concentration of 3 to 5 mM. All ascorbate-containing samples were dark-adapted at room temperature for 2 min (20 min without (2,6-dichlorophenol)indophenol (DCPIP)) before freezing in darkness for further treatment. Illumination of EPR tubes at 200 K was performed with a tungsten–halogen lamp (white light intensity ≈ 200 mW/cm^2) in a nitrogen gas flow system (Bruker, B-VT-3000). Light-induced warming of the tubes was minimized by filtering the light with a water cuvette and an infrared absorbing filter. The following procedure was followed for illumination at 200 K: samples were transferred in room light from liquid nitrogen to the gas flow system, kept in room light for 1 min for temperature equilibration, illuminated for a time period, then cooled down to 120 K within the gas flow system before being transferred to liquid nitrogen for later EPR measurements. This procedure allowed to avoid any temperature excursion above 200 K during sample manipulation that could lead to uncontrolled decay of signals. For experiments involving dark incubation at 200 K, the time period was considered to start just after transfer of the EPR tube from liquid nitrogen to the gas flow system at 200 K. This leads to an overestimation of the dark periods at 200 K because of the time needed for temperature equilibration of the sample. L-[1-^{13}C]Ascorbate and L-[2-^{13}C]ascorbate (99% enrichment) were from Omicron Biochemicals, Inc. (South Bend, IN, USA).

A reference $P700^+$ signal was obtained with a sample containing methylviologen and without ascorbate. This sample was illuminated for 30 s at room temperature and frozen into liquid nitrogen under illumination (moderate white light intensity, ≈ 40 mW/cm^2). EPR characterization of the sample revealed the presence of a single signal due to $P700^+$. Illumination inside the cavity did not lead either to any increase in the radical signal size or to the formation of any reduced iron–sulfur cluster. Therefore, this sample was considered to contain one $P700^+$ radical per PSI and was used as a reference for spin quantitation of radicals. Spin quantitations of radicals were performed under conditions of non-saturating microwave power (50 K, microwave power 2 μW).

A reference (F_A^-, F_B^-) signal was obtained with a sample containing 5 mM dithionite at pH 8.0. The sample was

illuminated at room temperature for 1 min at weak white light intensity (≈ 10 mW/cm^2) and frozen into liquid nitrogen under ambient light conditions. EPR characterization of the sample revealed the presence of the coupled spin system due to F_A and F_B being both reduced. Illumination inside the cavity at 20 K did not modify the signal. This sample was considered to contain 2 spins of reduced iron–sulfur centers and was used as a reference for spin quantitation of iron–sulfur clusters, which were measured at 20 K under conditions of non-saturating microwave power.

2.3. ESEEM spectroscopy

Pulsed EPR experiments were performed with a Bruker ESP380 spectrometer equipped with an Oxford Instruments cryostat. 2D-HYSCORE spectra were recorded using the sequence ($\pi/2-\tau-\pi/2-t_1-\pi-t_2-\pi/2-$echo), where the echo was measured as a function of t_1 and t_2. The durations of the $\pi/2$ and π pulses were 16 and 32 ns, respectively, with equal amplitudes. Two hundred and forty points were recorded in each dimension, while t_1 and t_2 were incremented in steps of 16 ns from their initial values. Fourier transforms of the time domain data were done after: (1) subtraction of a background cubic function, (2) apodization by a Hamming function and (3) zero filling.

3. Results

3.1. Illumination of PSI at 200 K produces an ascorbyl radical

Fig. 1 shows EPR spectra recorded at 50 K in the radical region when PSI reaction centers from the cyanobacterium *Synechocystis* sp. PCC 6803 were illuminated under different conditions. PSI samples were treated with ascorbate and DCPIP before freezing in darkness so that P700 was reduced and the electron acceptors oxidized before the light treatment. Trace b shows the spectrum which is obtained after illumination of PSI for 30 min at 200 K, followed by incubation in darkness for 10 min at the same temperature. The P700$^+$ spectrum, obtained by illumination at 50 K, is shown for comparison in trace a. Traces c and d were obtained when the 200 K treatment was performed with samples containing [1-^{13}C] and [2-^{13}C]ascorbate, respectively. Using a *g*-value of P700$^+$ of 2.0026 as a reference, the *g*-values (calculated from a first moment analysis of the absorption spectra obtained by integration of the experimental spectra) are 2.0045, 2.0042 and 2.0043 for traces b, c and d, respectively. The comparison between samples containing ^{12}C and ^{13}C ascorbate shows that the radical species formed at 200 K originates from ascorbate and that there is a significant spin density on carbons 1 and 2 of the ascorbyl radical.

The amount of photoaccumulated ascorbyl radical was quantified as a function of time of illumination at 200 K. This is shown is Fig. 2 for two samples with and without

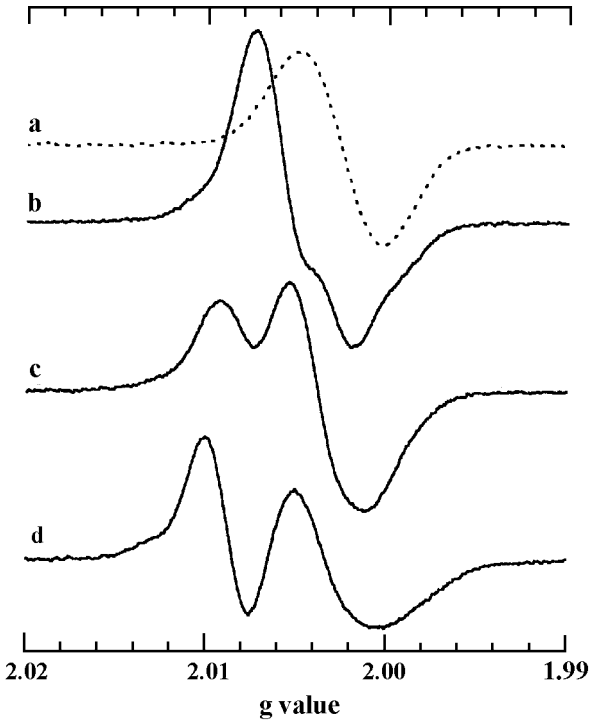

Fig. 1. X-band EPR spectra of PSI radicals formed in the presence of ^{12}C and ^{13}C ascorbate. EPR samples contained PSI from *Synechocystis* 6803 (1 mg chl/ml) in Tricine buffer 20 mM pH 8.0, with 4 mM potassium ascorbate and 40 μM DCPIP. EPR radicals were measured either after 1-min illumination at 50 K (trace a) or after 30-min illumination at 200 K (traces b–d). For traces b–d, illumination was followed by 20-min incubation in darkness at 200 K. Traces a and b were recorded with L-[^{12}C]ascorbate whereas traces c and d were recorded with L-[1-^{13}C]ascorbate and L-[2-^{13}C]ascorbate, respectively. Instrument settings: temperature 50 K, microwave frequency 9.43 GHz, microwave power 20 μW, modulation amplitude 0.2 mT.

DCPIP. The reference for spin quantitation was a PSI sample with P700 being fully oxidized (see Materials and methods). In the sample containing DCPIP, two ascorbyl radicals per PSI were photoaccumulated whereas a maximum of about 1.35 radical was observed in the absence of DCPIP. The line shapes were found to be identical whether DCPIP is present or not. In the presence of DCPIP, the kinetics of ascorbyl radical formation were fitted as a single or biexponential rise (see figure legend). The monoexponential fit is poor whereas the biexponential fit appears very good with identical amounts of the fast (t_{12} = 69 s) and slow (t_{12} = 424 s) components. In the absence of DCPIP, a satisfactory fit is obtained with a single rising component (t_{12} = 319 s). The ascorbyl radical is stable for hours in darkness at 200 K (not shown) whereas it decays in minutes at 220 K (Fig. 2).

3.2. Mechanism of ascorbyl radical formation

One simple way of explaining the formation of the ascorbyl radical at 200 K would be electron transfer from ascorbate to P700$^+$. This appears to be the right explanation

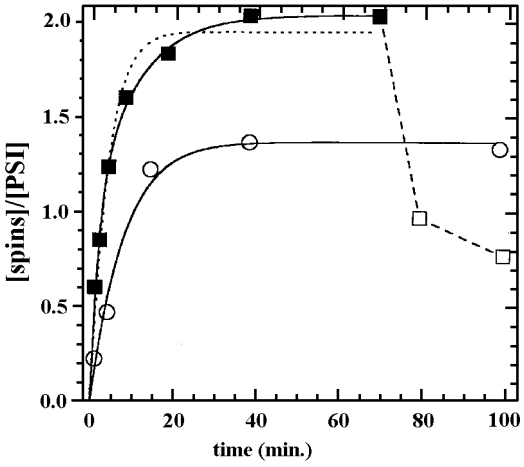

Fig. 2. Kinetics and spin quantitation of the ascorbyl radical formation as a function of illumination time at 200 K. The EPR samples contained PSI from *Synechocystis* 6803 (1 mg chl/ml) in Tricine buffer 20 mM pH 8.0, with 3 mM sodium ascorbate, either in the absence of DCPIP (circles) or in the presence of 30 μM DCPIP (squares). After each period of illumination, the sample was maintained for 60 min in darkness at 200 K before measurement in darkness. After 75-min illumination at 200 K, the DCPIP containing sample was incubated at 220 K (open squares). EPR measurements were performed at 50 K under conditions of non-saturating microwave power. The amounts of spins were quantitated by reference to a PSI sample containing 100% of $P700^+$ (see Materials and methods). The kinetics of radical formation was fitted either with a single exponential rise $A \times (1 - \exp(-kt))$ (closed squares, dotted line: $A = 1.95$, k corresponding to $t_{1/2} = 158$ s; circles, continuous line: $A = 1.37$, k corresponding to $t_{1/2} = 319$ s) or with a biexponential rise $A_1 \times (1 - \exp(-k_1 t)) + A_2 \times (1 - \exp(-k_2 t))$ (closed squares, continuous line, $A_1 = 1.01$, $A_2 = 1.03$, k_1 and k_2 corresponding to $t_{1/2} = 69$ and 424 s, respectively). Instrument settings as in Fig. 1 except microwave power 2 μW.

as shown by the experiment reported in Fig. 3. The starting spectrum (left part, dotted line) was measured with a PSI sample containing ascorbate and DCPIP which had been first illuminated for 3 min at 200 K and kept for 60 min in darkness at the same temperature. This led to the photoaccumulation of about one ascorbyl radical per PSI (dot spectrum; see the time dependence of photoaccumulation in Fig. 2). The sample was then submitted to a 16-min period of illumination at 200 K and frozen down to 120 K under illumination, before the measurement at 50 K (spectrum a). Later on, the sample was kept for 2 min in darkness at 200 K, before cooling for measurement at 50 K (spectrum b). The difference between a and b is shown in the right part of the figure (high noise spectrum a−b). Comparison of the resulting spectrum with the $P700^+$ spectrum (low noise superimposed spectrum, after amplitude adjustment) shows clearly that only $P700^+$ decay was occurring in the radical region during the 2-min dark period at 200 K. The sample was then submitted to successive dark periods of 10, 23 and 40 min at 200 K, with a 50 K dark spectrum being measured after each dark period. Spectrum c was measured after the last dark incubation period (i.e. a cumulative time of dark adaptation of 75 min). The difference between spectra c and b is shown in the right part of the figure (high noise spectrum). The superimposed low noise spectrum corresponds to a difference spectrum between the ascorbyl radical and $P700^+$ with identical amounts of spins (taking 1 per PSI), which was then multiplied by a factor of 0.2 for amplitude normalization. The quasi-identity between the two spectral shapes reveals that, between 2 and 75 min, the only detectable reaction was reduction of $P700^+$ by ascorbate. From the above spectra, the amount of ascorbyl radical which was formed is plotted in the higher right part of the figure as a function of the cumulative time of dark incubation at 200 K. As this ascorbyl radical was formed solely through $P700^+$ reduction, it shows that the halftime of the process is about 10 min. One can notice that the real process may be slightly faster as the dark adaptation time was measured starting from the time that the sample was transferred from liquid nitrogen to the nitrogen gas system which was used for temperature control at 200 K. One can also notice that these measurements correspond to the photoaccumulation of the second ascorbyl radical. Therefore, within the above experimental uncertainties, the 10-min halftime agrees reasonably well with the 7-min halftime that was determined for the slower phase of the ascorbyl radical formation.

It should be also emphasized that the ascorbyl radical was observed in spinach digitonin particles containing about 220 chlorophylls per P700, even though the stoichiometry was not checked in this case. An experiment was also performed with ascorbate/DCPIP and 1 mM benzoquinone which was added in ethanol solution (2.5% final amount, v/v) with PSI from *Synechocystis*. The control sample with 2.5% ethanol and no benzoquinone exhibits the same behavior as a sample without ethanol. In the sample with benzoquinone, it was not possible to photoaccumulate any ascorbyl radical and only $P700^+$ was observed in the radical region. This suggests that the PSI site which binds ascorbate is occupied by benzoquinone with a greater affinity.

The experiments depicted in the present paper are dealing with fresh samples that were never thawed after being illuminated at 200 K. Thawing a sample which had accumulated two ascorbyl radicals led to full disappearance of the radical signal (not shown). When such a sample was used to repeat the photoaccumulation experiments at 200 K, rise of the ascorbyl radical was slower and the stoichiometry was smaller (typically 1.1 to 1.5 spins per PSI). This behavior was not changed if ascorbate and DCPIP were added again after thawing. These observations suggest that thawing after formation of ascorbyl radicals leads to some partial irreversible reactions(s) between the radical moiety and PSI. Such an explanation is consistent with the binding of ascorbate at specific locations of PSI, as discussed below.

3.3. EPR properties of the ascorbyl radical

One could ask the question why such a radical was not previously reported as it can be formed in large amounts and seems easily detectable. First, most of the experiments involving illumination of PSI at 200 K were performed

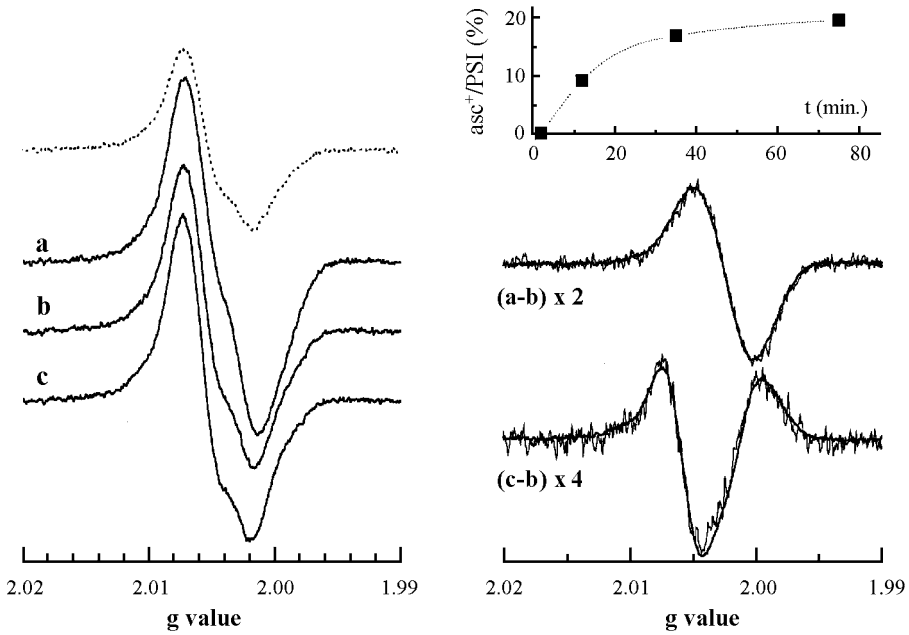

Fig. 3. Radical decay and formation in darkness after illumination at 200 K. The EPR samples contained PSI from *Synechocystis* 6803 (1 mg chl/ml) in Tricine buffer 20 mM pH 8.0, with 3 mM sodium ascorbate and 30 μM DCPIP. Left part: the four spectra were recorded with the same sample after the following consecutive treatments: dotted spectrum, 3-min illumination and 60-min dark incubation at 200 K; spectrum a, 16-min illumination at 200 K and cooling down to 120 K under illumination; spectrum b, 2-min darkness at 200 K; spectrum c, 73-min darkness at 200 K (three consecutive periods of 10, 23 and 40 min; cooling to 50 K for measurements after each period of dark incubation). Right lower part: the difference a−b (high noise spectrum) is compared to the full $P700^+$ low noise spectrum, which was multiplied by a factor of 0.44; the difference c−b (high noise spectrum) is compared to the difference (ascorbyl radical − $P700^+$) (1 spin equivalent of each per PSI; low noise spectrum), which was multiplied by 0.196. Right upper part: the kinetics of ascorbyl radical formation is shown as a function of the dark incubation time at 200 K, from spectra equivalent to spectrum (c−b). Instruments settings as in Fig. 2 (temperature 50 K, non-saturating microwave power).

under highly reducing conditions with dithionite and without ascorbate. Another answer to this question could be derived from the data of Fig. 4, which show that the ascorbyl radical saturates with microwave power much more easily than the $P700^+$ radical. Traces a, b and c were obtained at 60 K and at microwave powers of 2, 20 and 200 μW. These spectra were obtained with ascorbate and deuterated PSI from the cyanobacterium *Synechococcus* 7002 after 10-min illumination and 30-min dark incubation at 192 K. The width of the deuterated $P700^+$ signal is 0.4-mT wide (0.3 mT at room temperature, [24]), which makes it easily observable in the presence of large amounts of the ascorbyl radical. To compare the spectral shapes, spectra b and c were multiplied by factors of 0.42 and 0.3, respectively, so that the $P700^+$ signals appear equivalent. In spectra b and c, $P700^+$ is clearly saturated as spectra b and c should appear 1.3 and 3 times larger than spectrum a, if not saturated (taking into consideration the multiplicative factors used for displaying the data). However, the ascorbyl radical is clearly much more saturated than $P700^+$ as its low feature exhibits a significant decrease relatively to $P700^+$ at increasing microwave powers.

The contribution of $P700^+$ to spectrum a, which corresponds approximately to spectrum p, was subtracted for exhibiting the line shape of the ascorbyl radical (spectrum a−p). This is shown in the lower part of Fig. 4 together with the spectrum which is recorded with protonated PSI (dotted line). The sizes were adjusted so that the low-field positive peaks were superimposed. Two comments can be made from these observations: First, deuteration of PSI modifies the spectral shape of the ascorbyl radical, with a narrower negative peak. This is ascribed to a hyperfine coupling involving a deuteron which could be due either to a hydrogen bond to a PSI deuteron or to a chemical exchange of deuteron(s) and proton(s) during or after radical formation. Second, in deuterated PSI, the area of the negative peak is smaller than the area of the positive peak so that integration of the full radical signal does not give a proper absorption signal. This means that there is a significant field-dependent saturation (i.e. a field-dependent relaxation) of the radical with the high-field part of the spectrum being much more saturated than the low-field part. Under identical EPR conditions, the ascorbyl radical in protonated PSI (dotted spectrum) is not saturated, which points to slower relaxation in deuterated PSI. This effect may be ascribed to a weaker spin-phonon coupling between the radical and the PSI matrix when the matrix is deuterated.

3.4. HYSCORE of the ascorbyl radical

As described above, a small change in the CW-EPR spectrum of the ascorbyl radical was detected when deuter-

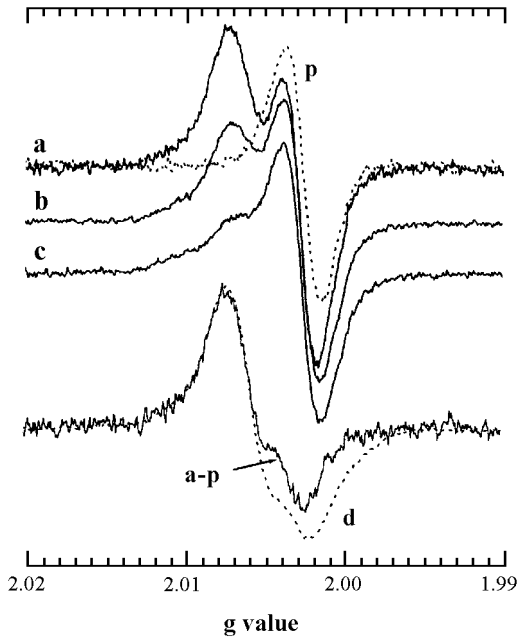

Fig. 4. X-band EPR spectra of the ascorbyl radical in deuterated PSI. The EPR sample contained PSI from *Synechococcus* 7002 (1 mg chl/ml) in Tricine buffer 20 mM pH 8.0, with 5 mM sodium ascorbate and 50 μM DCPIP. Cells were grown in A+ medium containing 95% D_2O and PSI was then purified in protonated water. All the following treatments were performed in protonated media and with protonated chemicals. The sample was illuminated for 10 min and dark-adapted for 30 min at 200 K. Spectra a, b and c were recorded in darkness at microwave powers of 2, 20 and 200 μW, respectively. The spectra are displayed after amplitude normalization of the 0.4-mT-wide $P700^+$ contributions. The approximate contribution of $P700^+$ in spectrum a is shown as a dotted spectrum p and is subtracted to give the spectrum (a−p) in the bottom part of the figure. This can be compared to spectrum d (dotted line) which is recorded in protonated PSI. The low-field peaks of spectra (a−p) and d are normalized to the same amplitudes. Instruments settings: temperature 60 K, microwave frequency 9.43 GHz, modulation amplitude 0.2 mT.

ated PSI was used instead of protonated PSI. This change must be attributed to non-exchangeable deuterium as deuterated PSI was studied in H_2O. Interactions between the ascorbyl radical and PSI were further investigated using HYSCORE spectroscopy, as this pulsed-EPR technique is particularly suitable for studying protons weakly coupled to paramagnetic centers, e.g. through hydrogen bonds (see, for example Refs. [25,26] and references therein). HYSCORE spectra have been recorded either with protonated PSI (Fig. 5A) or with deuterated PSI (Fig. 5D), both samples being prepared in ^1H_2O with protonated ascorbate. Measurements were done at the magnetic field corresponding to the maximum intensity of the ascorbyl radical absorption spectrum. Preliminary experiments had shown that the addition of sodium ascorbate resulted in a strong peak at the Larmor frequency of ^{23}Na, i.e. 3.94 MHz. To avoid this problem, the ascorbate salt was prepared from ascorbic acid with KOH.

Fig. 5A shows the (+, +) quadrant in which protons weakly coupled to the ascorbyl radical can be detected. These peaks are positioned symmetrically around 14.74 MHz (i.e. the nuclear Larmor frequency ν_i of ^1H) both on the $\nu_1 = -\nu_2$ off-diagonal axis and in an up-shifted position relatively to this off-diagonal axis. Data presented in Fig. 5 were obtained with a τ value equal to 136 ns, but similar results have been obtained with different τ values (not shown). The ^1H-peaks almost completely disappeared when the ascorbyl radical was formed in the presence of deuterated PSI (Fig. 5D). The proton peaks are replaced by peaks situated around 2.2 MHz as expected for hyperfine couplings between an $S = 1/2$ electronic spin and the $I = 1$ nucleus spin of deuterium. In Fig. 5D the remaining weak proton peaks at (12.6, 17.3 MHz) might be due either to matrix protons, or to a specific proton with a small isotropic coupling $A_{iso} \approx 4.7$ MHz. Due to the lack of additional spectroscopic information, the chemical nature of this proton is actually difficult to discuss further. The presence of ^1H in deuterated PSI may be due either to deuterons exchanged for protons during PSI purification or incomplete deuteration during culture growth.

More interestingly, comparison of the HYSCORE spectra in Fig. 5A and D demonstrate that the ^1H characterized by peaks, up-shifted from the $\nu_1 = -\nu_2$ off-diagonal axis by $\Delta\nu_{max}^{shift} \approx 0.65 \pm 0.05$ MHz (Fig. 5A and B), is a PSI proton, which therefore provides a hydrogen bond donor to the ascorbyl radical. From Fig. 5A, it is possible to estimate both the anisotropic (T) and the isotropic (A_{iso}) hyperfine couplings of this proton, following an analysis given in Refs. [25,26]. The shape of the HYSCORE arcs is indicative of an axial hyperfine coupling, with a $T_\perp$ value of its anisotropic component which can be deduced from the off-diagonal shift $\Delta\nu_{max}^{shift}$. $\Delta\nu_{max}^{shift}$ was precisely determined from a slice 1D spectrum, obtained at the maximum of the out-of-diagonal cross-peak (at 16.8 MHz in the vertical (ν_1) dimension; Fig. 5B). From the relation $T_\perp = 2/3((8\Delta\nu_{max}^{shift}\nu_i)/\sqrt{2})^{1/2}$ with $\Delta\nu_{max}^{shift} \approx 0.65$ MHz and $\nu_i = 14.74$ MHz, we estimate $T_\perp \approx 4.9$ MHz. Using this value with the point-dipole approximation, we estimate the distance between this proton and the ascorbyl radical to ≈ 2.5 Å (see, e.g. Ref. [27]). This is certainly a very rough estimation which assumes the point-dipole approximation with 100% spin density on the ascorbyl radical oxygen involved in the hydrogen bond.

Together with $T_\perp$, the frequency distance d between the points where the inner end of the arcs touch the diagonal $\nu_1 = -\nu_2$ can be used to estimate the isotropic coupling A_{iso} (Fig. 5A). A precise value of d was obtained from a 3D plot of the HYSCORE data (Fig. 5C). We then use the relation $d = A_{iso} - T_\perp$ for calculating $A_{iso} \approx 6.9$ MHz.

3.5. Multiple photoreduction of iron–sulfur clusters at 200 K

Spectrum a (continuous line) of Fig. 6 shows the EPR spectrum of reduced iron–sulfur clusters F_A and F_B that was observed when an ascorbate containing PSI sample was illuminated at 200 K for 30 min and cooled down to 120 K

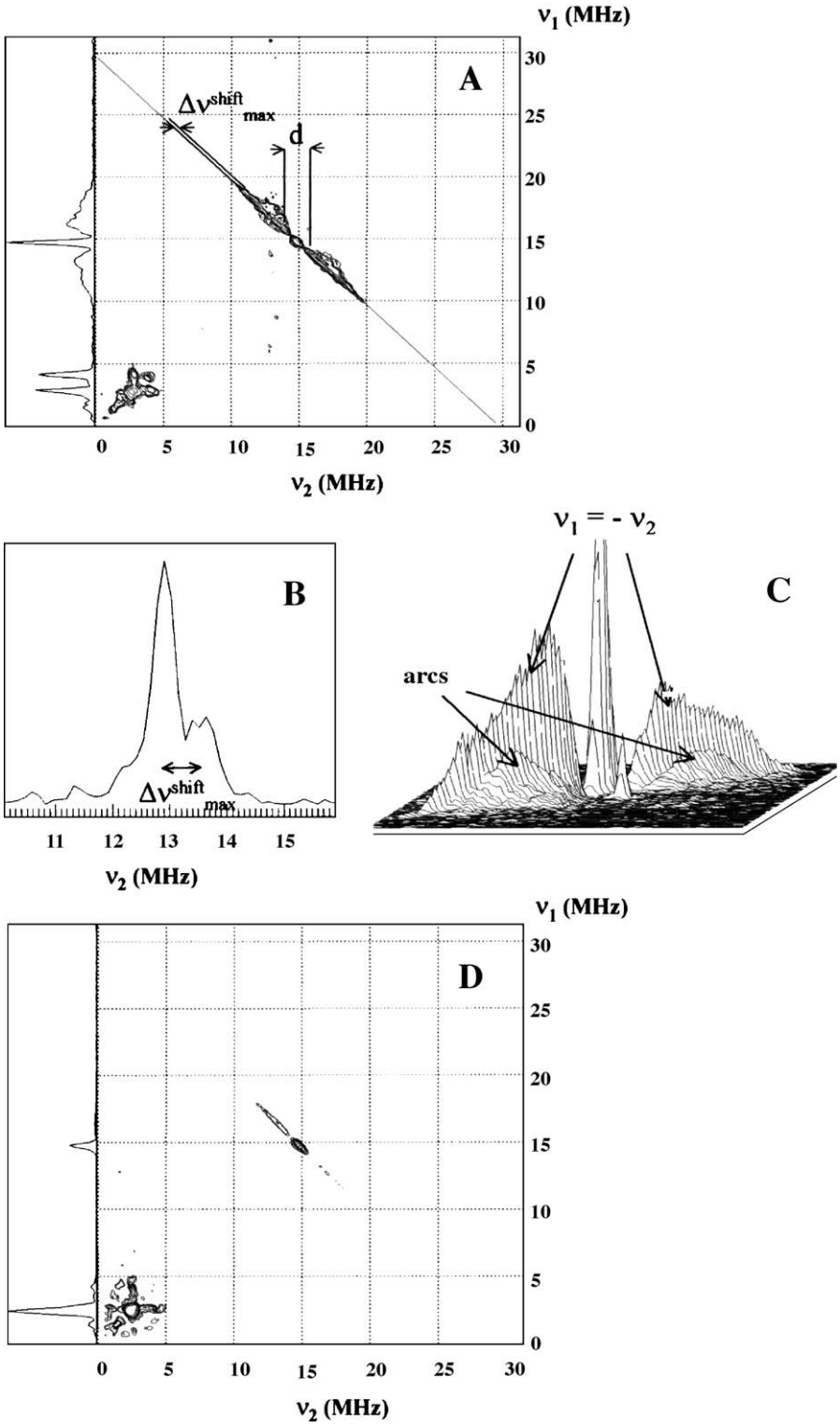

Fig. 5. HYSCORE spectra of the ascorbyl radical. Contour plots of the frequency domain HYSCORE spectra of the ascorbyl radical formed in the presence of either protonated PSI (Panel A) or deuterated PSI (Panel D). Panel B shows a slice projection from Panel A done at 16.8 MHz in the vertical (ν_1) dimension. Panels A and D also show the skyline projection of the 2D-spectra. Panel C is a 3D plot of the same HYSCORE spectra displayed in panels A and B. It shows the data above the $\nu_1 = -\nu_2$ diagonal with the on-diagonal protons and the arcs. Experimental conditions: magnetic field, 3449 gauss (maximum of the absorption spectrum); temperature, 4 K; microwave frequency 9.66 GHz; shot repetition time, 40 ms; $\tau = 136$ ns, $T_{min} = 40$ ns, 240×240 points with a 16 ns step; Larmor frequency, 14.74 MHz.

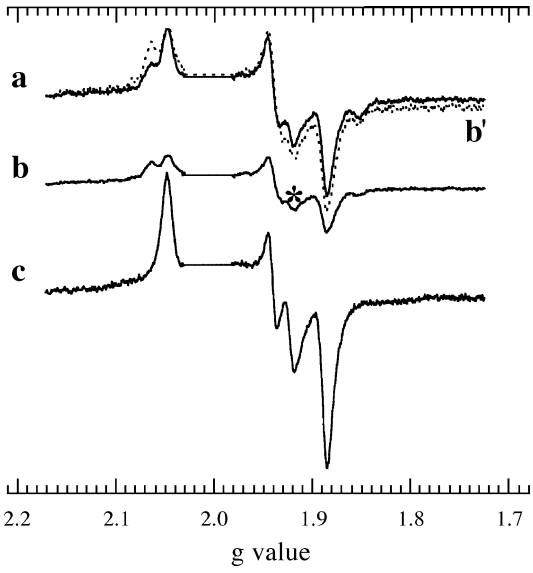

Fig. 6. Reduction of both iron–sulfur clusters F_A and F_B by illumination at 200 K. The EPR samples contained PSI from *Synechocystis* 6803 (1 mg chl/ml) in Tricine buffer 20 mM, pH 8.0. Spectra a and b were measured with a PSI sample containing 3 mM sodium ascorbate and 30 μM DCPIP. Spectrum a was obtained after 30-min illumination at 200 K and temperature lowering down to 120 K under illumination. Thereafter, the sample was maintained for 20 min in darkness at 200 K before recording spectrum b. For line shape comparison with spectrum a, spectrum b was multiplied by a factor of 2.4 and is displayed is the upper part of the figure (spectrum b′ , dotted line). Spectrum c: after addition of 10 mM sodium dithionite, the sample was illuminated for 30 s at room temperature with weak light before being frozen in liquid nitrogen in room light. Microwave power was not saturating the F_A/F_B signals. Signals in the radical region were erased for display and spin quantitation. Relative spin amounts of iron–sulfur clusters are 1.19, 0.72 and 2.00 for spectra a, b and c, respectively. Instruments settings: temperature 20 K, microwave frequency 9.42 GHz, microwave power 2 mW, modulation amplitude 1 mT.

under illumination. Spectrum c is a control spectrum with a sample in which F_A and F_B were both completely photoreduced in the presence of dithionite. Illumination of this sample at 20 K gave no further signal, thus confirming the complete reduction of both F_A and F_B, with the characteristic spectral features of the spin-coupled (F_A^-, F_B^-) system (*g*-values at 2.048, 1.941, 1.923 and 1.886). Counting 2 spins per PSI reaction center in spectrum c, spectrum a counts for 1.19 spins. A number of spins larger than 1 indicates that, in a fraction of PSI, multiple turnover occurred which resulted in F_A and F_B being both reduced. This can be seen also by the presence of the 1.923 resonance which is observed only in the spin-coupled system [F_A^-, F_B^-] [28]. Multiple turnover was expectable in view of the above observations that $P700^+$ can be reduced by ascorbate, which then allows for a second photochemical charge separation (and a third one after formation of two ascorbyl radicals). The spectral features at $g = 1.855$ and 2.065 correspond to PSI reaction centers with singly reduced F_A^- and F_B^-, respectively. When the sample was maintained in darkness for 20 min at 200 K (spectrum b), the signal amplitude decreased down to 0.72 spin equivalent. However, the small feature around 1.92 (asterisk in Fig. 6) indicates the persistence of a small (F_A^-, F_B^-) signal. The signal decrease from spectrum a to spectrum b can be ascribed, at least partially, to a recombination reaction of the reduced clusters with $P700^+$, according to many previous reports (see, e.g. Ref. [29]) and is in line with the $P700^+$ decay reported in Fig. 3 (spectrum a–b). This is also seen by comparing the line shapes of spectra a and b. For this comparison, spectrum b was multiplied (by a factor of 2.4) and superimposed with spectrum a (dotted line; spectrum b′). When comparing spectra a and b′ , one can see that most lines are of similar size with the notable exception of the low-field line at 2.065 (due to F_B^-) which is much larger in spectrum b′ . Some other minor differences are also observable, one of which being a small decrease of the high-field line at 1.855 (due to F_A^-) in spectrum b′ compared to spectrum a. These differences indicate that F_A^- has decayed faster than F_B^- in darkness, which may be rationalized in terms of distances, F_A^- being closer to $P700^+$ than F_B^- and therefore recombining faster with $P700^+$. In a sample identical to the one studied here (spectrum a), almost 2 spin equivalents of the ascorbyl radical was formed after prolonged illumination. Comparing spin amounts due to the radicals and the iron–sulfur clusters shows that electron escape from the clusters to an exogenous acceptor, presumably oxygen, must occur at 200 K. F_B^- is probably primarily responsible for this process as it is much more exposed to the solvent [4].

3.6. Photoreduction at 200 K produces a modified F_B^-

When PSI was submitted at 200 K to a 2-min period of illumination followed by 10 min in darkness, the EPR spectrum recorded at 20 K (spectrum b of Fig. 7) reveals the presence of a minor F_A^- signal (*g*-values = 2.047 and 1.853) and a major F_B^- signal (*g*-values = 2.065, 1.938 and 1.884, vertical lines). For comparison, the 20 K light-induced spectrum obtained by illumination of another dark-pretreated sample is shown in spectrum a. The usual spectral features of isolated spins due to F_A^- (*g*-values = 2.047, 1.944 and 1.855) and F_B^- (*g*-values = 2.068, 1.931 and 1.881) can be recognized. The spectrum of F_B^- after illumination at 200 K deviates significantly with the one formed after illumination at 20 K. The deviations qualitatively agree with those previously observed in spinach particles which were attributed to temperature-dependent conformational changes [30]. When comparing the *g*-values of F_A^-, no significant difference could be observed between spectra after illumination at 20 or 200 K, contrary to the case of spinach for which the low and high-field *g*-values were both shifted to slightly higher values upon illumination at 200 K (taking 20 K illumination as a reference) [30]. A pure spectrum of the 200 K F_B^- spectrum was tentatively obtained by subtracting small amounts of spectrum a

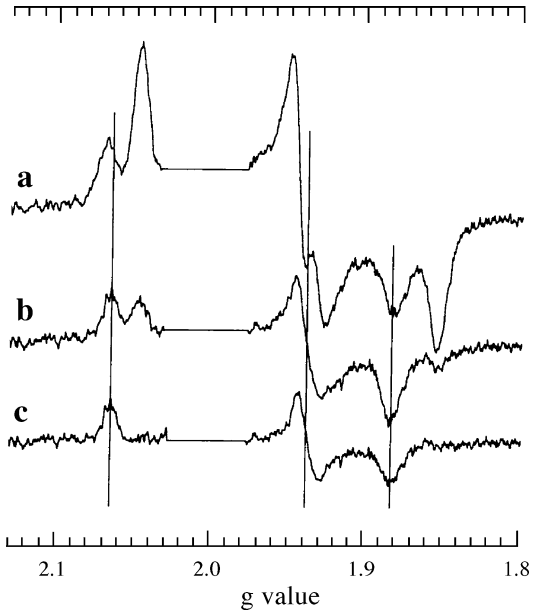

Fig. 7. Modified form of F_B^- after illumination at 200 K. PSI from *Synechocystis* 6803 (1 mg chl/ml) in Tricine buffer 20 mM pH 8.0, with 3 mM sodium ascorbate and 30 μM DCPIP. Spectrum a: the sample was cooled down to 20 K in darkness and illuminated for 1 min at 20 K. The light-induced signal is exhibited. Spectrum b: the sample was illuminated for 2 min and kept in darkness for 10 min at 200 K. Spectrum c was obtained by subtracting 14% of spectrum a and 4% of a fully reduced (F_A^-, F_B^-) spectrum (spectrum c of Fig. 6). Vertical lines correspond to *g*-values of 2.065, 1.938 and 1.883. Microwave power was not saturating the F_A/F_B signals. Signals in the radical region were erased for display and spin quantitation. Relative spin amounts of iron–sulfur clusters are 0.93, 0.31 and 0.12 for spectra a, b and c, respectively, by taking a reference value of 2.00 for spectrum c of Fig. 6. Instrument settings as in Fig. 6.

(mostly F_A^-) and of spectrum c of Fig. 6 (F_A^-, F_B^-). The resulting spectrum of modified F_B^- is shown in spectrum c of Fig. 7 (*g*-values = 2.065, 1.938, 1.883).

4. Discussion

Illumination of PSI reaction centers at 200 K in the presence of ascorbate is shown to result in the formation of a radical, the occurrence of which has never been reported before in studies of PSI. EPR experiments performed with ^{13}C-labelled ascorbate show that this radical originates from ascorbate (Fig. 1). Direct evidence has been obtained that the radical is formed upon reduction of $P700^+$, the primary donor of PSI (Fig. 3), by ascorbate, following an illumination period at 200 K. The *g*-value of the ascorbyl radical is estimated at 2.0044 ± 0.0003, using a reference *g*-value of 2.0026 for $P700^+$. Spin quantitation in the presence of DCPIP shows that a maximum of two radicals per PSI is found after a long period of illumination. This indicates that the radical is not formed in solution but arises from reactive ascorbate molecules which are bound to PSI at specific positions prior to freezing. We tentatively speculate that the stoichiometry of 2 is related to the pseudo-C_2 symmetry axis of PSI. This symmetry axis is perpendicular to the membrane, relates the large hydrophobic subunits PsaA and PsaB and goes through the middle of P700 and the iron–sulfur center F_X. Therefore, we propose that PsaA and PsaB both bind one ascorbate at positions which are approximately symmetrical to the C_2 axis. Assuming a symmetrical positioning, the kinetics of formation can be tentatively interpreted by considering that each side is associated with different kinetics with either fast or slow donating ascorbate molecules (named «fast» and «slow» in the following). When DCPIP is absent, only 1.35 ascorbyl radicals per PSI is formed and the kinetics of radical formation is slower and similar to the kinetics observed for the «slow» ascorbate in the presence of DCPIP. This effect is puzzling and no detailed explanation is fully satisfactory: whether DCPIP is needed only for the «fast» ascorbate or DCPIP greatly accelerates the formation of both ascorbyl radicals, why is there 1.35 photoaccumulated radicals instead of 1? As no new species is formed with DCPIP, it can be concluded anyway that DCPIP acts only as a helper, playing, e.g. a role as an intermediate electron carrier between P700 and ascorbate.

The existence of a specific ascorbate binding site is also strongly supported by the EPR data when comparing the spectra of the radical prepared with either protonated or deuterated PSI, in the presence of protonated water and buffer in both cases. First, the line shape of the radical is narrower with deuterated PSI than with protonated PSI, thus indicating a significant hyperfine coupling to a non-exchangeable PSI deuteron (proton) (Fig. 4). Second, this coupling is seen by HYSCORE spectroscopy (Fig. 5) which provides an upper limit of 2.5 Å for the distance between the PSI proton and the oxygen of the ascorbyl radical involved in the hydrogen bond. This PSI proton may originate from a non-exchangeable NH or OH group. For being non-exchangeable, such a group should be either buried or involved in a strong hydrogen bond. Its interaction with the water-soluble ascorbate molecule would mean that ascorbate binding involves some structural rearrangement of the PSI binding site. Alternatively, a CH group might be a good candidate for providing the non-exchangeable proton, in line with previous reports of C–H···O hydrogen bonds (see, e.g. Refs. [31,32]), for which (C–)H···O distances between 2.2 and 2.7 Å have been reported. An isotropic hyperfine coupling constant $A_{iso} \approx 6.9$ MHz was also determined from the HYSCORE data. To our knowledge, such a high value of a hyperfine coupling, which should involve some covalent character of the hydrogen bond, is unprecedented in the case of a protein hydrogen bonded to a radical. A covalent character of hydrogen bonds has been documented in the case of strong hydrogen bonds called low-barrier hydrogen bonds [33–35]. However, such bonds involve only N and O as heteroatoms and a C–H···O hydrogen bond is not expected to have a significant covalent character. The existence of a large isotropic hyperfine coupling would be therefore more consistent with the PSI heteroatom being N or O, the N(or O)–H···O bond having some small covalent character. There-

fore, the covalent character, favoring a N(or O)–H· · ·O bond, and the non-exchangeability, favoring a C–H· · ·O bond, of the hydrogen bond that we observe, appear at first sight to be contradictory. Further work is clearly required to identify the exact nature of this hydrogen bond.

Different ascorbyl radicals have been characterized by EPR either in solution at room temperature or in single crystals at low temperature with various *g*-values between 2.0033 and 2.0052 (see, e.g. Refs. [36–39]). The doublet that is usually observed in biological samples at room temperature has a *g*-value of 2.0052 and is probably a deprotonated anion radical with the spin density being mostly delocalized on carbons 1, 2 and 3 [36]. The *g*-value of the present radical is significantly shifted compared to this value and further studies and calculations are needed to clarify its exact nature. Formation at low temperature in a rather homogeneous biological environment may be helpful for its further characterization.

The reduction of $P700^+$ by ascorbate proceeds rather slowly at room temperature and this process is not considered to be physiologically relevant, although it constitutes the most common way of pretreating PSI for in vitro studies. Ascorbate is rather abundant in chloroplasts (12–25 mM, [40]). It is probably primarily located in the stroma where it is a substrate for ascorbate peroxidase [17] and plays a key role in defence against oxidative stress. To our knowledge, its presence in the lumen has not been reported and it is not expected to cross the thylakoid membrane in its anionic form ($pK \approx 4$; [18]). Its content in cyanobacteria is much lower (20–100 μM, [41]). Various amounts of ascorbate peroxidase activities have been observed in cyanobacterial cell extracts [42,43], but this activity has been estimated to be unimportant in cyanobacteria due to the small in vivo ascorbate concentration [43]. Our data are clearly not dealing with the in vivo state of things and we consider that $P700^+$ reduction of ascorbate at 200 K is of no particular biological significance. However, it may be useful to recognize it as a side effect which should be known for in vitro studies and as a potentially useful artefact in PSI studies at low temperature. To our knowledge, this is also the first report of a homogeneous ascorbyl radical being trapped in an enzyme for low temperature studies.

The reactions which occur in PSI reaction centers in the presence of ascorbate under illumination at 200 K are discussed in the following, while comparing our results to those previously published. (1) Light absorption leads to charge separation between $P700^+$ and one of the two terminal iron–sulfur clusters F_A^- or F_B^- (see Introduction). (2) A recombination reaction occurs between $P700^+$ and $(F_A, F_B)^-$, with highly heterogeneous kinetics in the seconds to hours time domain [29]. In an experiment depicted in Fig. 3, $P700^+$ decays just after illumination within 2 min in darkness at 200 K. This decay is due solely to recombination with reduced F_A^- or F_B^-, as no ascorbate signal contributes to the spectrum difference. However, only reduction of $P700^+$ by ascorbate is occurring between 2 and 75 min in darkness after illumination, without any involvement of a recombination reaction. This shows that the recombination reaction is over after 2 min. This behavior contrasts with the wide distribution of kinetics observed in Ref. [29], which may be related to the use of glycerol. (3) We find in the present work that ascorbate can reduce $P700^+$ at a very small rate, which allows multiple turnover of PSI. Such a multiple turnover is generally assumed not to occur in the presence of ascorbate. From the present experiments, this assumption appears to be correct inasmuch as the period of illumination is short enough: a few seconds with DCPIP, a few tens of seconds in its absence, with PSI from *Synechocystis* 6803. In a previous study which makes use of illumination of PSI at different temperatures, the absence of multiple turnover has been explicitly mentioned [30]. We propose that some of the spectra which were obtained in the latter study contain contributions of the spin-coupled system (F_A^-, F_B^-) formed by multiple turnover. However, our data confirm one essential conclusion of that paper, i.e. the spectral shape of F_B^- depends on the temperature of photoreduction (Fig. 7): after dark adaptation following a short illumination period, a spectrum dominated by the high-temperature form of F_B^- was obtained with wild-type PSI in the absence of glycerol (Fig. 7). This spectrum is different from the one obtained by illumination at 10–25 K, in line with Ref. [30]. In our case and contrary to this last study, no significant difference was observed concerning F_A^-, whatever the temperature of photoreduction is (20 or 200 K). In view of the known structure of PSI with cluster F_B being relatively more exposed to the solvent and thus in a potentially more flexible environment, this can be rationalized by assuming that reduction of F_B leads to some structural relaxation of the F_B^- environment which is possible only at 200 K. In the case of F_A, relaxation following reduction could be restricted because of structural constraints associated with its position farther from the surface. (4) The ascorbyl radicals are stable at 200 K but decay at 220 K. The partner of the ascorbyl radical in this reaction was not identified. (5) Escape of electrons from reduced iron–sulfur clusters (presumably to oxygen) is also occurring, as is shown by the amount of reduced iron–sulfur clusters which is smaller than the amount of photoaccumulated ascorbyl radicals.

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